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Identification of a pentatricopeptide repeat RNA editing factor in *Physcomitrella patens* chloroplasts

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ABSTRACT

The moss *Physcomitrella patens* has two RNA editing sites in the chloroplasts. Here we identified a novel DYW-subclass pentatricopeptide repeat (PPR) protein, PpPPR_45, as a chloroplast RNA editing factor in *P. patens*. Knockdown of the *PpPPR_45* gene reduced the extent of RNA editing at the chloroplast *rps14*-C2 site, whereas over-expression of *PpPPR_45* increased the levels of RNA editing at both the *rps14*-C2 site and its neighboring C site. This indicates that the expression level of PpPPR_45 affects the extent of RNA editing at the two neighboring sites.

1. Introduction

In plants, RNA editing frequently occurs at specific cytidines (C) to convert uridines (U) in organelle transcripts. Thirty to 40 RNA editing sites have been identified in chloroplasts and over 500 sites in mitochondria of flowering plants [1,2]. Some editing events create translation initiation codons and therefore RNA editing seems to be an essential process for organelle biogenesis [3,4]. However, the molecular mechanism of RNA editing is not completely understood.

Genetics and biochemical studies over the last decade have revealed that nuclear-encoded pentatricopeptide repeat (PPR) proteins are involved in RNA editing in plant organelles [4,5]. PPR proteins are widely distributed among protists, yeasts, animals and plants [6] and play a central role in the post-transcriptional and translational regulation in mitochondria and chloroplasts [7,8]. Plant-specific PPR proteins with a C-terminal E or E and DYW domains site-specifically recognize target RNA editing sites and perform RNA editing [5]. In addition, general editing factors such as RNA binding proteins [9,10] MORF/RIP proteins [11,12], and protoporphyrinogen IX oxidase 1 (PPO1) [13] participate in RNA editing in *Arabidopsis* organelles.

In contrast to flowering plants, the moss *Physcomitrella patens* has only 11 editing sites in the mitochondria [14,15] and eight DYW-subclass PPR proteins have been identified as editing site specific recognition factors at all 11 sites [15–20]. On the other hand, two editing sites have been identified in the *P. patens* chloroplasts [21]. Editing at the *rps14*-C2 site occurs at a high efficiency and creates a translation initiation codon AUG. In addition, the *rps14*- -1C site in the 5′ untranslated region (UTR) is edited at a low efficiency (~5%) [21]. These editing sites also exist in the related moss *Funaria hygrometrica* [17], but not found in the chloroplasts of higher plants. However, no editing factors for these sites have been identified yet.

Here, we report that a DYW-subclass PPR protein, PpPPR_45, is required for RNA editing at the two sites in the chloroplast *rps14* transcript.

2. Materials and methods

2.1. Subcellular localization of PpPPR_45 fused to green fluorescent protein (GFP)

Isolation of RNA from *P. patens* protonemata, preparation of RNA-free cDNA and amplification of cDNA fragments by polymerase chain reaction (PCR) were carried out as described previously [22]. The amplified cDNA encoding the N-terminal 118 amino acids of PpPPR_45 was cloned in-frame into the *Sma*I site in pKSPGFP9

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[15]. The resultant plasmid p45N-GFP was introduced into the transgenic Mt-RFP OX moss line, which expresses the mitochondria-localized red fluorescent protein (RFP) [20]. GFP fluorescence was monitored using a confocal microscope FLUOVIEW FV10i (Olympus).

2.2. Selection of RNA interference (RNAi) and over-expression lines

RNAi moss line selection was performed according to [23]. A target 0.6 kb DNA region encoding the 16th to the 22nd PPR motifs of PpPPR_45 was introduced into pGG626 RNAi vector by the Gateway LR reaction and p45RNAi was generated. To construct the over-expression p45OX plasmid, the *PpPPR_45* coding region was amplified from cDNA using the primers listed in Supplementary Table 1. The PCR product was cloned into the *Swa*I site of the modified pOX7WH1, which was derived from the over-expression vector pPpMADS2 7113 containing the E7113 promoter (containing seven 35S promoters and additional enhancers [24]), a NOS terminator and an *nptII* cassette. The *nptII* cassette was replaced with the hygromycin resistance gene (*hpt*) cassette in pOX7WH1. The linearized plasmids, p45RNAi and p45OX, were introduced into the GFP-tubulin/histone-RFP moss line (GH line, [23]) or the wild type, respectively, and hygromycin-resistant lines were selected. To analyze RNAi mosses, the protonemata were cultured in the presence of 1 μM β-estradiol.

2.3. Chlorophyll fluorescence analysis

Chlorophyll fluorescence was measured with a kinetics multispectral fluorescence imaging FluorCam 800 MF (Photon System Instruments) according to the manufacturer's instructions. Thirty-day-old protonemal colonies were dark-adapted for 10 min before measurement.

2.4. RNA analysis

Quantitative (q) reverse transcription (RT)-PCR was carried out as described previously [20] using the primers indicated in Supplementary Table 1. *TUA1* (α-tubulin) was used as the internal control. RNA editing efficiency was calculated from the number of edited cDNA clones. For RNA gel blot analysis, gene-specific DNA of the chloroplast *rps14* (311 bp) was amplified using the primers in Supplementary Table 1, and was internally labeled with digoxigenin as described previously [22].

3. Results

3.1. PpPPR_45 is localized in the chloroplasts

DYW-subclass PpPPR_45, deduced from the gene (Pp1s543_6V6.1, http://www.cosmoss.org [25]) and cDNA (AB979874), was predicted to be localized in the chloroplasts and therefore was expected to be a candidate of the RNA editing factor for the chloroplast *rps14* transcript [26]. PpPPR_45 consists of 23 PLS-type PPR motifs and C-terminal E/E+ and DYW domains (Fig. 1A). To verify the chloroplast localization, we performed a transient expression assay of PpPPR_45-GFP protein. GFP fluorescence was observed in the chloroplasts but not in the mitochondria nor in the nucleus (Supplementary Fig. 1), indicating that PpPPR_45 is a chloroplast-localized protein.

3.2. Knockdown of PpPPR_45 resulted in growth retardation and a reduction of photosynthetic activity

To investigate whether PpPPR_45 is involved in RNA editing of the chloroplast *rps14* transcript, we initially tried to generate *PpPPR_45* knockout mosses by insertion of an *nptII* or *hpt* gene cassette into the second exon of the gene. However, no knockout mosses were obtained from the genotyping of 251 moss plants. Instead, we generated *PpPPR_45* RNAi mosses by introducing an inducible RNAi construct into the transgenic GH moss line [23]. Among several RNAi candidate lines selected, two lines (45RNAi #3 and 45RNAi #5) reduced the level of *PpPPR_45* mRNA as measured by qRT-PCR (Fig. 1B). The two independent RNAi lines exhibited retarded growth when treated with β-estradiol. In addition, the pale-green phenotype was observed on the surface

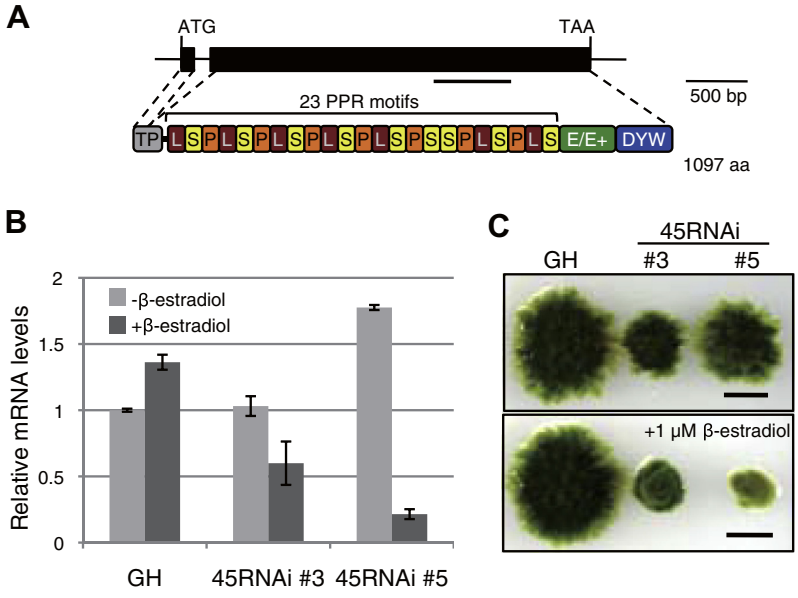

Fig. 1. Generation and characterization of the *PpPPR_45* RNAi lines. (A) Schematic structure of the *PpPPR_45* gene and the encoded protein. Black boxes indicate the translated regions. The amplified region using RNAi is underlined. TP indicates a putative transit peptide. (B) qRT-PCR analysis to quantify *PpPPR_45* mRNA levels in GH and 45RNAi lines with or without β-estradiol. SDs are indicated ($n = 3$). (C) Protonemata colonies of GH and 45RNAi lines. The mosses were grown for 30 days on BCDATG medium plates with or without 1 μM β-estradiol. Bars = 10 mm.

of protonemata colonies grown for 30 days (Fig. 1C). Therefore, we measured photosynthetic activities by pulse amplitude modulation. The quantum yield of electron transfer through PSII (φPSII) decreased to 75% and 8% in the induced 45RNAi lines #3 and #5, respectively, compared with the non-induced GH line. In addition, the maximum (Fv/Fm) and effective (Fv′/Fm′) quantum yields were also reduced, but photochemical quenching (qP) did not change (Supplementary Table 2). This indicates that the electron transport from PSII to PSI was not affected, but that the efficiency of PSII or accumulation of active PSII complexes was reduced in the induced 45RNAi plants.

3.3. PpPPR_45 is involved in RNA editing of the chloroplast rps14 mRNA

We then investigated RNA editing of the chloroplast *rps14* transcript in the 45RNAi plants. For this analysis, more than 50 independent *rps14* cDNA clones were randomly isolated and sequenced. In the GH line, RNA editing at the *rps14*-C2 site occurred at 65% either with or without β-estradiol treatment. In contrast, the level of RNA editing decreased to 16.7% (9 edited/54 cDNA clones) and 2% (1 edited/51 clones) in the induced RNAi lines #3 and #5, respectively (Fig. 2, lower panel). However, a clear change in the editing level at the *rps14*- -1C site could not be observed because the extent of editing of this site was less than 10% even in the GH line (Fig. 2, upper panel). A secondary cause of this low editing efficiency may be due to aberrant RNA processing. To investigate this possibility, the steady-state level and pattern of the *rps14* transcript were analyzed by an RNA gel blot. The data shows that there were no obvious alterations to the *rps14* transcript in the induced or non-induced RNAi lines (Supplementary Fig. 2). The 45RNAi and GH plants did not affect the 11 editing sites in the mitochondria (data not shown). This indicates that the reduction of RNA editing in the induced RNAi lines is due to a direct effect of PpPPR_45.

To confirm the involvement of PpPPR_45 in *rps14* editing, we further generated *PpPPR_45* over-expression mosses (45OX#6, #114, and #203 lines, Fig. 3). The protonemata of the 45OX lines grew slower and had fewer gametophores than the wild type (Fig. 3A). RT-PCR analysis showed that the expression levels of *PpPPR_45* increased considerably (Fig. 3B). We then sequenced 20 independent *rps14* cDNA clones. In the wild type and the vector control line, 65% and 68% RNA editing, respectively occurred at *rps14*-C2. In contrast, the extent of RNA editing exceeded 90% in the 45OX lines, reaching 100% in the 45OX#6 line. Furthermore, the level of RNA editing extent at the *rps14*- -1C site increased to 33% and 27% in the 45OX lines #6 and #114, respectively (Fig. 3C).

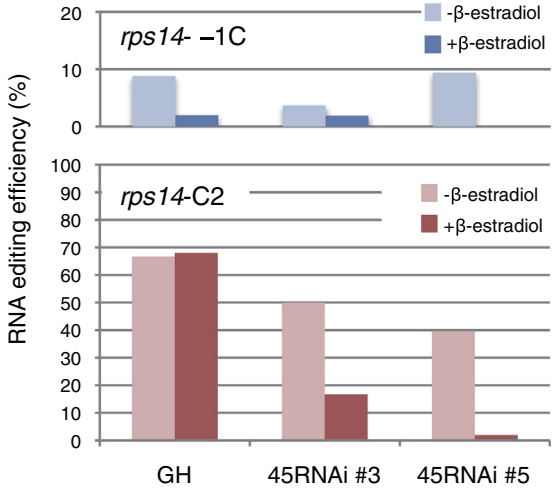

Fig. 2. RNA editing of chloroplast *rps14* transcript in *PpPPR_45* RNAi lines. The levels of RNA editing at the *rps14*-C2 site (lower panel) and the *rps14*-−1C site (upper panel) from sequencing of more than 50 independent cDNAs in GH and 45RNAi lines. Mosses were grown for 4 days with or without 1 μM β-estradiol.

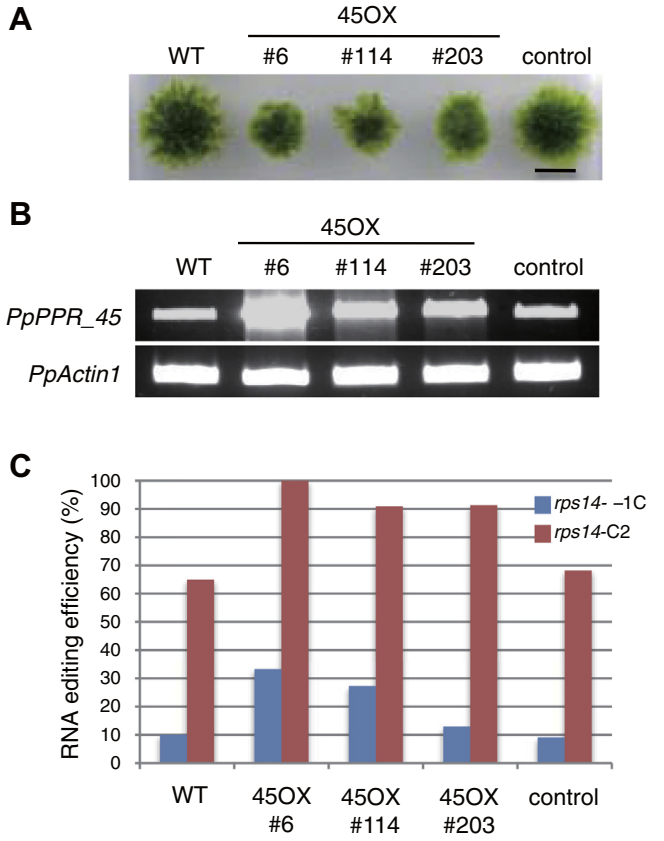

Fig. 3. RNA editing of chloroplast *rps14* transcript in *PpPPR_45* over-expression lines. (A) Visible phenotype of the wild-type (WT), 45OX lines and the vector control line introduced the empty pOX7WH1 (control). Mosses were grown for 15 days on BCDAT medium plates without antibiotics. Bars = 10 mm. (B) RT-PCR was performed to detect cognate transcripts. *PpActin1* transcript was used as the control. (C) The levels of RNA editing at the *rps14*-C2 and −1C sites after sequencing 20 independent cDNAs.

4. Discussion

In this study, we showed that DYW-subclass PpPPR_45 is involved in RNA editing at the two neighboring sites in the chloroplast *rps14* transcript. In *Arabidopsis* mitochondria, there are several neighboring editing sites, e.g. *nad1*-307 and *nad1*-308 sites [27]. MEF25 is essential for RNA editing at *nad1*-308 but not at *nad1*-307 [27]. This indicates that these contiguous editing sites are recognized by different factors. In contrast, the two neighboring *rps14*-C2 and −1C sites can be recognized by the same protein, PpPPR_45. A question is how PpPPR_45 recognizes at both C sites and perform their RNA editing. Recently, amino acid codes for RNA recognition by PPR proteins have been elucidated [28–30]. Previous alignments of PPR editing factors with their target sites have indicated that the PPR proteins bind with the C-terminal PPR motif aligned with the nucleotide at −4 with respect to the edited C [30]. Thus, there is usually a strict dependence between an editing site and the exact position of the aligned PPR editing factor. According to the amino acid code, we predicted the target RNA sequence of PpPPR_45 and aligned the predicted sequence with two target sites for PpPPR_45 (Fig. 4). This alignment leads us to

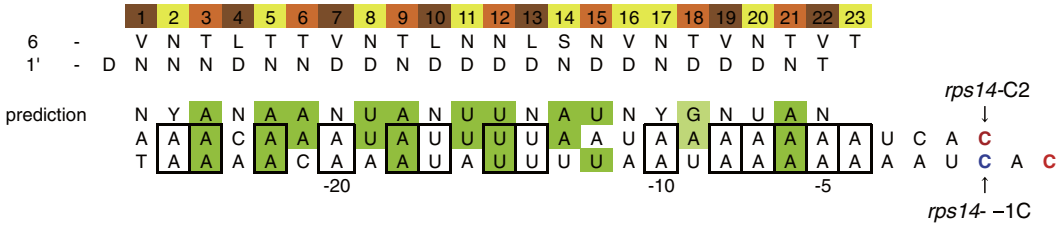

Fig. 4. Alignment of PpPPR_45 to its target sites. Target sequence prediction was carried out as described in [28–30]. The amino acids at position 6 and 1′ in PPR motifs are indicated. Position 1′ is the first amino acid of the respective C-terminally adjacent repeat. The target nucleotide sequences are shown in red or blue (*rps14*-C2 and -1C, respectively) for PpPPR_45. Light green shading indicates a significant correlation between the prediction and target RNAs. Matched nucleotides among two target RNAs are boxed.

consider two alternative binding modes for PpPPR_45, one with the binding site from the positions -25 to -4 relative to the *rps14*-C2 site and the other with the binding site shifted 2 nucleotides upstream. These two binding sites are A/U-rich and share 64% nucleotide identity. The low-level editing at *rps14*- -1C might be considered "accidental" editing due to an alternative binding of PpPPR_45 with the neighboring two C targets.

In *P. patens* chloroplast *rps14*, the translation initiation codon is encoded by ACG in the genome, which is altered to AUG by RNA editing at the *rps14*-C2 site. *rps14* encodes a ribosomal protein S14, a component of the small 30S ribosomal subunit. In *Escherichia coli*, RPS14 is essential for cell viability and is required for the assembly of the 30S ribosomal subunits [31]. It is suggested that the reduction of RNA editing in the *rps14* initiation codon impaired the translation of RPS14 protein and affected the function of the chloroplast ribosome. This prediction may result in a pale-green phenotype and inhibited photosynthetic activity in the induced 45RNAi plants. The efficiency of *rps14*-C2 editing is controlled in a tissue- and stage-specific manner [21]. In addition, the growth rates of protonemata and gametophore were delayed in the both 45RNAi and OX mosses. These observations suggest that RNA editing at the *rps14*-C2 site regulates the translation of chloroplast proteins and plant development. *rps14* has another editing site at the -1 position relative to the translation initiation codon, but the efficiency was less than 10% in the wild type. On the other hand, over-expression of PpPPR_45 resulted in an increase in the level of editing at the *rps14*- -1C site. RNA editing at the *rps14*- -1C site may affect the efficiency of *rps14* translation. This question remains to be addressed.

Our previous and present study revealed that all editing events in *P. patens* chloroplasts and mitochondria require DYW-subclass PPR proteins [15,16,18,20]. Thus, *P. patens* now is the first organism with a complete assignment of specificity factors to all its organellar editing sites. DYW domains contain a highly conserved motif HxE. . .CxxC similar to cytidine deaminase [32]. In vivo complementation analysis demonstrated that this conserved motif in the DYW domain of DYW1 is essential for RNA editing of the *ndhD*-1 site in *Arabidopsis* chloroplasts [33]. In addition, *P. patens* has neither E-subclass PPR proteins nor MORF/RIP proteins. This leads us to consider that the editing machinery comprises the simplest editosome in *P. patens*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.09.031.

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